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The characterization of the *Phlebotomus papatasi* transcriptome

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Abstract

As important vectors of human disease, phlebotomine sand flies are of global significance to human health, transmitting several emerging and re-emerging infectious diseases. The most devastating of the sand fly transmitted infections are the leishmaniases, causing significant mortality and morbidity in both the Old and New World. Here we present the first global

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Authors' Contributions: JA designed and performed all of the bioinformatic analyses. MRO isolated RNA from all samples and coordinated sample shipment to ExpressGenomics for library construction and aided in experimental design. PL maintained the *P. papatasi* colony and carried out blood feeding and sampling of life-cycle stages. SK organized and performed quality control of the adult and larval samples used for library generation. EDR oversaw the maintenance of the sand fly colony. MRO, MJB, PAB, JGV, FHC, RD and MAM conceived the project, were authors on the white paper that funded the project, and participated in overall experimental design. CT, EA, EM, SC, and RKW were involved in various stages of sequencing and analysis. RD provided *L. longipalpis* sequences. NFL, FHC, and MAM advised the bioinformatic analysis. SE and SO performed the heterozygosity analysis. DS, GS and JA performed the qPCR analysis. MW and MB maintain the *Ph. papatasi* colony and collected samples. MAM managed all activities.

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14. ABSTRACT

As important vectors of human disease, phlebotomine sand flies are of global significance to human health, transmitting several emerging and re-emerging infectious diseases. The most devastating of the sand fly transmitted infections are the leishmaniases, causing significant mortality and morbidity in both the Old and New World. Here we present the first global transcriptome analysis of the Old World vector of cutaneous leishmaniasis, Phlebotomus papatasi (Scopoli) and compare this transcriptome to that of the New World vector of visceral leishmaniasis, Lutzomyia longipalpis. A normalized cDNA library was constructed using pooled mRNA from Phlebotomus papatasi larvae, pupae, adult males and females sugar fed, adult females blood fed and fed blood infected with Leishmania major. A total of 47,615 generated sequences were cleaned and assembled into 17,120 unique transcripts. Of the assembled sequences, 50% (8,837 sequences) were classified using Gene Ontology (GO) terms. This collection of transcripts is comprehensive, as demonstrated by the high number of different GO categories. An in depth analysis has revealed 245 sequences with putative homology to proteins involved in blood and sugar digestion, immune response and peritrophic matrix formation. Twelve of the novel genes, including one trypsin, two peptidoglycan recognition proteins (PGRP) and nine chymotrypsins have a higher expression level during larval stages. Two novel chymotrypsins and one novel PGRP are abundantly expressed upon blood feeding. This study will greatly improve the available genomic resources for Ph. papatasi and will provide essential information for annotation of the full genome.

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transcriptome analysis of the Old World vector of cutaneous leishmaniasis, *Phlebotomus papatasi* (Scopoli) and compare this transcriptome to that of the New World vector of visceral leishmaniasis, *Lutzomyia longipalpis.* A normalized cDNA library was constructed using pooled mRNA from *Phlebotomus papatasi* larvae, pupae, adult males and females sugar fed, adult females blood fed and fed blood infected with *Leishmania major*. A total of 47,615 generated sequences were cleaned and assembled into 17,120 unique transcripts. Of the assembled sequences, 50% (8,837 sequences) were classified using Gene Ontology (GO) terms. This collection of transcripts is comprehensive, as demonstrated by the high number of different GO categories. An in depth analysis has revealed 245 sequences with putative homology to proteins involved in blood and sugar digestion, immune response and peritrophic matrix formation. Twelve of the novel genes, including one trypsin, two peptidoglycan recognition proteins (PGRP) and nine chymotrypsins have a higher expression level during larval stages. Two novel chymotrypsins and one novel PGRP are abundantly expressed upon blood feeding. This study will greatly improve the available genomic resources for *Ph. papatasi* and will provide essential information for annotation of the full genome.

Introduction

Phlebotomine sand flies are important vectors of human disease in both the Old and the New World, transmitting protozoan, bacterial and viral pathogens. These flies are members of the family Psychodidae, which includes a diverse group of vectors that vary widely in geographic distribution, ecology, and the pathogens they transmit. Sand flies serve as vectors for several established, emerging and re-emerging infectious diseases, the most devastating of which are the leishmaniases with 350 million people at risk and approximately two million new cases each year (Cunningham 2002, Desjeux 1996). Human migration, political instability, and warfare is expanding *Leishmania*-endemic regions and increasing the propensity for epidemics world-wide (Desjeux 2001). In spite of the medical importance of leishmaniasis it is classified as a neglected tropical disease and phlebotomine sand fly species remain understudied.

Approximately 40 different species of *Leishmania* are transmitted by 35 different sand fly species (Ramalho-Ortigao, Saraiva & Traub-Cseko 2010). Most vectors belong to one of two genera, *Phlebotomus* and *Lutzomyia* (Richard P. Lane, Roger W. Crosskey 1993). *Phlebotomus* species are responsible for transmitting leishmaniasis throughout parts of Africa, southwest Asia, the Middle East, and the Mediterranean basin; *Lutzomyia* species are vectors throughout the Americas. There is a close ecological relationship between *Leishmania* species and the vector(s) that transmit that species. For example, *Phlebotomus papatasi* only transmits *Leishmania major* whereas *Lutzomyia longipalpis* transmits *Le. infantum chagasi* (Killick-Kendrick 1999). Although most vectors are specific under natural conditions, some, such as *Lu. longipalpis*, can transmit a range of *Leishmania* species under laboratory conditions.

Genomics approaches enable comprehensive comparisons between diverse organisms, thus facilitating a more complete understanding of their biology. The completed genomes of the more widely studied hematophagous vectors, the malaria vector *Anopheles gambiae*, the yellow fever mosquito *Aedes aegypti*, and the West Nile virus vector *Culex quinqefasciatus*, have already contributed valuable information relative to vectorial capacity, blood-feeding, insect immune system modulation and insecticide resistance (Holt et al. 2002, Christophides et al. 2002, Nene et al. 2007, Arensburger et al. 2010). Previous genomic studies concerning sand flies have focused on specific questions regarding vector-human interactions (analysis of protein expression in salivary glands (Hostomska et al. 2009)) or vector-parasite interactions (analysis of midgut expressed proteins (Ramalho-Ortigao et al. 2007)); only one study has performed a global gene discovery analysis of a sand fly, *Lu. longipalpis* (Dillon

et al. 2006). Here, we expand these studies by characterizing the transcriptome of the *Le. major* vector, *Ph. papatasi*. We also similarly reanalyze the *Lu. longipalpis* Expressed Sequence Tag (EST) dataset (Dillon et al. 2006) for a comparative analysis.

Phlebotomine sand flies along with mosquitoes (family Culicidae) are members of the suborder Nematocera, but these two families are representatives of distinct infraorders within the Nematocera. *Ph. papatasi* and *Lu. longipalpis* exhibit distinct geographical distributions, ecology, and vector competence specificity. A comparative approach between these flies will accelerate the discovery of regulatory and biochemical pathways within this family as potential biopharmaceuticals, vaccine candidates, and targets for insecticide development. Moreover, comparative analyses between these and other available vector data sets will elucidate the pathways that lead to arthropod blood-feeding and immunity and inform arthropod phylogenetic relationships.

ESTs represent the expressed portion of mRNA in a cell obtained through single pass sequencing of randomly selected cDNA clones, resulting in about 200-800 bp of sequence information for each clone (Mark Blaxter et al. 2009). EST studies can be used for gene discovery in organisms where sequencing the whole genome is not possible (Lindlof 2003), or in addition to genome information for more accurate gene annotation. A phlebotomine sand fly genome sequencing project was initiated a few years ago to sequence *Lu. longipalpis* and *Ph. papatasi* (McDowell et al. 2006). The aim of this study was to increase the availability of EST resources for future sand fly studies, provide useful information regarding the biology of these important vectors, and generate essential data for annotation of the newly sequenced phlebotomine sand fly genomes (McDowell et al. 2006).

Results and Discussion

Assembly

Mate pair information generated during the sequencing process was utilized in a two step assembly process. First, the sequence reads were assembled with their mates using a lower identity criteria based on the assumption that they are opposite ends of the same cDNA clone and thus should assemble. Resulting sequences were assembled using a more stringent identity parameter to avoid over collapsing closely related gene families into a single sequence. There were 47,615 sequences initially generated from the normalized *Ph. papatasi* library. Of all initial *Ph. papatasi* reads, 10,128 (21%) failed the screening and filtering steps (see Experimental procedures) (Figure 1). The remaining 37,487 sequences were then assembled into 6,187 contigs and 10,933 singlets (Table 1), representing a total of 11 Mb of the *Ph. papatasi* transcriptome, average assembled sequence length was 550 bp. Assembled sequences with a length greater than 200 bp were deposited in GenBank (JP539097-JP555361).

A total of 3,909 (14%) *Lu. longipalpis* sequences failed the screening and filtering steps. The remaining cleaned sequences (24,019) were assembled into 5,063 contigs and 4,963 singlets, average length of 1,041 bp, representing a total of 8 Mb of the *Lu. longipalpis* transcriptome (Table 1). The differences in assembly results between the current study and the previous assembly by Dillon et al. (Dillon et al. 2006) can be explained by utilization of a different assembly program (Cap3 vs. Phrap) as well as by the different assembly strategy used (Supplementary Figure 1) (Huang, Madan 1999).

For our analyses we defined a read as a sequence that was cleaned and trimmed before the first assembly step. A mate pair represented two reads sequenced from the same cDNA; these reads had the same name with a different ending denoting either the 5′ or 3′ end. A mated read contig referred to mate pairs that co-assembled. A contig was either a mated read

or a set of sequences that were not mate pairs but assembled under the conditions indicated above. One contig represented one gene, unless otherwise specified. Singlets are sequences that failed to assemble with any other sequence in the set, including their own mate pairs.

Similarity to known proteins and GO annotation

Of the 17,120 *Ph. papatasi* assembled sequences, 4,286 (25%) had no matches when searched against the NR and InterPro databases using a BLAST search (BLASTX) with an e-value threshold of 10^{-5}, making them potentially unique *Ph. papatasi* sequences. The average length of assembled sequences with matches against either NR or InterPro database was between 500 and 699 bp long (Figure 2). Of the total assembled sequences, 8,837 (50%) of the *Ph. papatasi* and 4,411 (44%) of the *Lu. longipalpis* sequences could be associated with a GO term in at least one of the three main categories (biological process, molecular function or cellular component). The level of annotation is smaller than that for *Anopheles* mosquito (*Anopheles albimanus*, 65% (Martinez-Barnetche et al. 2012) and higher than non-model organisms with little or no closely related species in NCBI NR database (~17- 50%) (Du et al. 2012, Hou et al. 2011, Wang et al. 2010, Shen et al. 2011). The limited annotation may be explained by the lack of sequences for closely related species available in NR and InterPro.

The high number of different GO categories suggests that our cDNA library is representative of the entire organism. The distribution of GO categories was highly similar (Figure S2-4) between the two sand fly species, with *Ph. papatasi* having 35 (0.2%) sequences in four extra categories (viral reproduction, external encapsulating structure, symbiosis and neurotransmission categories). The smaller number of sequences available for *Lu. longipalpis*, combined with the small number of sequences annotated in the *Ph. papatasi* dataset, might account for their absence in the *Lu. longipalpis* dataset. Some of the differences were probably due to the original source of RNA prepared from the two species (the *Lu. longipalpis* RNA was extracted from adult females only, while for the *Ph. papatasi* RNA extraction, immature life stages and males were included) see Experimental procedures and Dillon et all. (Dillon et al. 2006). There were no GO categories present in *Lu. longipalpis* that were absent in the *Ph. papatasi* dataset.

The highest number of sequences in the biological process category for both sand flies were annotated as catabolic process (1,326, 15% - *Ph. papatasi;* 562, 12.7% - *Lu. longipalpis*). In the Molecular Function category, the majority of *Ph. papatasi* and *Lu. longipalpis* sequences were annotated as nucleotide binding (1,412 or 16%, and 719 or 16.3% respectively). For the Cellular Component category, a high number of sequences were annotated as protein complex (1,898, 21.4%; 864, 19.6%) (Figure 3).

Digestive proteins

Trypsin and chymotrypsin-like serine endoproteases involved in blood digestion have been characterized in mosquitoes and other blood feeding arthropods, and their expression level was associated with the type and the time elapsed since a blood meal acquisition (Ramalho-Ortigao et al. 2007, Muller et al. 1993, Noriega, Wells 1999, Pitaluga et al. 2009, Telleria et al. 2010, Vizioli et al. 2001). Serine proteases are also involved in many other key processes in insects including complex cascades of proteases involved in immune signaling pathways (Buchon et al. 2009). Serine proteases, like trypsin, are considered important in the interaction of the sand fly host with *Leishmania*; trypsin activities being modulated in the gut of both *Ph. papatasi* and *Lu. longipalpis.* Knockdown of a ‘late’ trypsin in *Lu longipalpis* enhanced the survival of *Le. mexicana* (Sant'Anna et al. 2009). Previously, four trypsins and three chymotrypsins in *Ph. papatasi* (Ramalho-Ortigao et al. 2007, Ramalho-Ortigao et al. 2003) and two trypsins and five chymotrypsins in *Lu. longipalpis* (Telleria et al. 2010,

Jochim et al. 2008) were identified. Here we identify five new trypsin-like sequences including PpTryp5a (JP544502) and PpTryp5b-e (JP542407, JP540627, JP554453, JP544448), that may represent alleles of PpTryp5a because they have an identity over 95% at the amino acid level (Figure 4A). PpTryp5a has a similarity of 32-39% at the amino acid level to known *Ph. papatasi* trypsins and a 50% identity to the closest related sequence in the NR database –the *Ae. aegypti* trypsin. The newly identified trypsin sequences (PpTrp5a-e) clustered closest to each other and to the *Ae. aegypti* trypsin rather than to any of the six previously identified sand fly trypsins (Figure 4B). This grouping may be explained by the difference in cDNA library sources, one possibility is that they are larval or pupal specific genes; previously the trypsin sequences were identified using a library constructed from the midgut of adult females only, while the library described here was constructed using whole sand fly bodies at different life stages that included both sexes. The *Ae. aegypti* trypsin sequence was identified from the entire genome sequence.

Seventeen novel chymotrypsin-like sequences with an identity of 29-69% to known *Ph. papatasi* chymotrypsin sequences were identified in the current study. Five putative chymotrypsin sequences PpChym4a,b,5-7 (JP546634, JP554565, JP551370, JP547341, JP554731) contained all the domains identified in other chymotrypsins (Ramalho-Ortigao et al. 2007, Ramalho-Ortigao et al. 2003, Appel 1986, Park, Kwak 2008) except for two amino acid differences in an otherwise highly conserved region. The first substitution, an S to F, is present only in PpChym4b (position 292) while the second substitution, a P to A, was present in all five sequences, PpChym4a,b-7 (position 293) (Figure 5A) . Four sequences representing two putative different chymotrypsins (PpChym14a and PpChym13; PpChym14b and PpChym14c are likely alleles of PpChym14a) cluster close to the previously identified PpChym2 (Ramalho-Ortigao et al. 2003), to which they share a 66, 67 and 69% identity at amino acid level. PpChym11 (including the 2 likely alleles: Ppchym11a and PpChym11b) and PpChym12 cluster closer to midgut chymotrypsin from *Glossina morsitans morstans* (Alves Silva et al. 2010). The last five of the novel putative chymotrypsin sequences (four of which are likely alleles of PpChym8 and PpChym9) cluster closer to chymotrypsin-like serine protease from *Chironomus riparius* expressed in the larvae gut (Park, Kwak 2008). The closer clustering of novel *Ph. papatasi* chymotrypsin sequences to sequences from different species could be explained by a possible difference in expression location (tissue) and/or life stage between the known and the novel chymotrypsin sequences; the known chymotrypsin sequences having been described in adult sand flies (Ramalho-Ortigao et al. 2007, Ramalho-Ortigao et al. 2003) (Figure 5B).

Aminopeptidases catalyze the removal of amino acids from the N-terminus of peptides and proteins. Their expression, along with the expression of exopeptidases, was shown to be modified by the ingestion of infected blood meal in sand flies (Muller et al. 1993, Dillon, Lane 1993). Our analysis identified 45 *Ph. papatasi* sequences with high identity to different aminopeptidases, including three aminopeptidase P sequences. Of these three sequences, only one (PpAPP1- JP547392) had all the necessary conserved domains (Kulkarni, Deobagkar 2002), with a 73% amino acid identity to *Ae. aegypti* proline specific aminopeptidase (e-value 10^{-127}). The other two sequences have a high identity to PpAPP1 (JP539747, JP552630) (over 98% identity spanning 100-144 amino acids) but lack one or more conserved domains. There were no aminopeptidase P sequences identified in *Lu. longipalpis*. This difference between the two species is more likely due to the nature of the datasets rather than the loss of the gene from *Lu. longipalpis*. We also have identified four sequences (JP41850, JP543532, JP547607, JP546724) with similarity to leucyl aminopeptidase, two of which are unassembled mate reads and all four of them have a higher than 97% identity at the amino acid level making them potentially alleles of the same sequence, PpAPL1 (JP541859). Four sequences with similarity to leucyl aminopeptidase were identified in *Lu. longipalpis,* three of which are probably allelic variants of LlAPL1a

which has 32% identity at the amino acid level to the leucyl aminopeptidase identified by Dillon et al. (Dillon et al. 2006) (Figure 6). It was not possible to confirm aminopeptidase identity (using conserved domain identification) in the remaining 39 *Ph. papatasi* sequences with homology to known aminopeptidases due to the short sequence length (76-585 AA). These *Ph. papatasi* sequences are likely to represent aminopeptidase transcript fragments rather than whole sequences (Supplemental Table2).

Carboxypeptidases also are enzymes integral to protein digestion in insects (e.g. mosquitoes) (Edwards et al. 2000). In sand flies, five sequences have been isolated from midgut, two carboxypeptidase B (one from each species) and three carboxypeptidase A (one from *Ph. papatasi* and two from *Lu. longipalpis*) (Ramalho-Ortigao et al. 2007, Pitaluga et al. 2009). Eighteen sequences (Supplemental Table2) related to carboxypeptidase A were identified in *Ph. papatasi*, these most likely represent transcript fragments. An additional two *Ph. papatasi* sequences (JP552630, JP546271) with similarity to known carboxypeptidase B were identified in this study. The new putative carboxypeptidase B sequences share a 38% and 54% identity, respectively, to known sand fly carboxypeptidases (Figure 7). While blood meals are a preferred source of protein for sand flies, they can survive on a sugar diet. Glucosidases are involved in carbohydrate digestion and their expression was shown to be modified by both sugar and blood meals in *Ph. langeroni* (Dillon, El Kordy 1997). Here we have identified 23 unique *Ph. papatasi* sequences with high identity (BLASTP, <1e-50) to known glucosidases from mosquitoes.

Chitin is an insoluble polysaccharide present in the insect cuticle, peritrophic matrix and the lining of the foregut, hindgut and trachea (Zhu et al. 2008). Chitinolytic enzymes are important for the periodical rearrangement and degradation of the exoskeleton and peritrophic matrix. To date, chitinases have been identified in many different arthropod species including fruit flies (*Dr. melanogaster*) (Zhu et al. 2004), mosquitoes (*Ae. aegypti, An. gambiae*) (De la Vega et al. 1998), silk moths (Koga et al. 1997), red flour beetles (*Tribolium castaneum*) (Zhu et al. 2008) and sand flies (*Ph. papatasi* and *Lu. longipalpis*) (Ramalho-Ortigao, Traub-Cseko 2003, Ramalho-Ortigao et al. 2005). The two known sand fly chitinases have been identified in the midgut and implicated in *Leishmania* – sand fly interactions (Ramalho-Ortigao, Traub-Cseko 2003, Ramalho-Ortigao et al. 2005). Seven more sequences with identity to the previously identified chitinase from *Ph. papatasi* (57%) and from related organisms, including *Ae. aegypti* and *Dr. melanogaster* (Ramalho-Ortigao et al. 2005), were identified in *Ph. papatasi* in the current study. However the search for conserved domains (Zhu et al. 2008) has revealed these sequences to be more likely transcript fragments rather than full-length chitinase sequences. Eight *Ph. papatasi* sequences related to amylase were identified in this study. Thirty-one sequences with high identity (BLASTP, <1e-50) to lipase also were identified in *Ph. papatasi* (Supp Table2).

Immune response Proteins

Insect immune responses have been most extensively studied in other dipteran insects including in *Dr. melanogaster* challenged with bacteria or fungi (Lemaitre, Hoffmann 2007) and in mosquitoes challenged with *Plasmodium* parasites (Cirimotich et al. 2010, Richman et al. 1997, Tahar et al. 2002) or bacteria (Dimopoulos et al. 1997, Hillyer, Schmidt & Christensen 2004). There have been only a few studies regarding the immune response of sand flies to parasite infection (Ramalho-Ortigao et al. 2007, Pitaluga et al. 2009, Jochim et al. 2008). Here we have identified *Ph. papatasi* sequences belonging to three of the four immune response pathways (Cirimotich et al. 2010, Tanaka et al. 2008), ranging from recognition receptors to effector proteins. There were no significant matches found to components of the JAK/STAT pathway in either sand fly.

Recognition proteins

Peptidoglycan recognition proteins (PGRPs) are highly conserved (Liu et al. 2001) across species that bind peptidoglycan present in the cell walls of bacteria. To date PGRPs have been identified in several arthropod species, including fruit flies (Werner et al. 2000), moths (Kang et al. 1998), silkworms (Tanaka et al. 2008, Ochiai, Ashida 1999), mosquitoes (Christophides et al. 2002) and sand flies (Pitaluga et al. 2009). Two new PGRP sequences were identified in *Ph. papatasi*, one is a short singlet with high identify to other sequence (PpPGRP2 –JP540873). PpPGRP2 has 44% identity to *Ae. aegypti* PGRP-LC and a 41% and 44% identity to known sand fly PGRPs (*Lu. longipalpis* and *Ph. papatasi,* respectively (Jochim et al. 2008)). Three additional sequences were identified with high similarity to PGRPs short class (PGRP-SC1-3, JP551327, JP547206, JP539467) (Figure 8).

β-glucan recognition proteins (βGRPs) are involved in recognition of Gram-negative bacterial and fungal cell walls and in triggering the prophenoloxidase (PPO) cascade (Yoshida, Ochiai & Ashida 1986, Ochiai, Ashida 2000). The βGRP class is composed of two functionally distinct proteins; one that binds β-1,3 glucan and one that binds Gram-negative bacteria. In the *Ph. papatasi* dataset we have identified three sequences with high similarity to βGRPs, two (JP543291, JP552580) that likely represent two alleles of the same sequence, PpBGRP1, whereas the third sequence (PpBGRP2- JP544368) is most likely a transcript fragment. These three novel βGRPs have amino acid identity to βGRPs from the pyralid moth *Plodia interpunctella* (37%) (Fabrick, Baker & Kanost 2003) and from the mosquito *Armigeres subalbatus* (44%) (Wang et al. 2005). Three sequences (JP542600, JP540290, JP541890) with high similarity to Gram-Negative Binding Proteins (GNBPs) were also identified. No new sequences with similarity to βGRPs were identified in the *Lu. longipalpis* dataset, LlBGRP1 and LlBGRP2 having previously been described by Dillon et al. (Dillon et al. 2006).

C-type lectins are a large protein family with low conservation at the amino acid level, they have diverse functions (Zelensky, Gready 2005), including pathogen recognition and neutralization (Weis, Taylor & Drickamer 1998). We have identified seven C-type lectins (PpCTL1-3) in the *Ph. papatasi* dataset with similarity to known C-type lectins from other insects. Two are alleles of PpCTL1a (JP543952, JP44832, JP547369) and one is an allele of PpCTL2a (JP543073, JP55083). PpCTL3a (JP554123) and PpCTL3b (JP540156) share a high identity at amino acid level (over 95%), however PpCTL3b is 139 amino acids longer than PpCTL3a. There were no sequences with high identity to known C-type lectins identified in *Lu. longipalpis*.

Three classes of the scavenger receptor protein family serve as recognition receptors in immune responses (Peiser, Mukhopadhyay & Gordon 2002, Kiefer et al. 2002, Pierini 2006, Ramet et al. 2001). Dillon et al. (Dillon et al. 2006) previously identified proteins belonging to two of the three classes (B and C) in *Lu. longipalpis*. Here we added six *Ph. papatasi* sequences with identity to class B scavenger receptors. Three of the new *Ph. papatasi* putative scavenger receptors (PpSR1b-d, JP543420, JP543780, JP539722) may represent alleles of PpSRB1a (JP541894). PpSRB1a-d and PpSRB2 (JP543210) share an identity of 29-31% to the known *Lu. longipalpis* scavenger receptors and 44-49% identity to *An. gambiae* scavenger receptor class B, while PpSRB3 (JP553019) has an identity of 96% to NSFM-84c11.q1k (SRB) from *Lu. longipalpis* and 80% to *An. gambiae* scavenger B receptor.

Galectins have been implicated in cell adhesion (Ochieng, Leite-Browning & Warfield 1998), apoptosis (Perillo et al. 1997), and immune response (Tanaka et al. 2008) and, for sand flies, in species-specific binding of *Leishmania* parasites to the sand fly midgut (Kamhawi et al. 2004). Seven sequences related to galectins were identified in *Ph. papatasi*.

One of these novel sand fly galectins is most likely an allele of the known *Ph. papatasi* galectin (PpGal1 (JP539532), previously identified as PpGalec [75]), four more sequences (PpGal2a-d, JP540648, JP546602, JP550066, JP540193) have less than 95% identity at the amino acid level to PpGal1, three of which (PpGal2b-d) are alleles of PpGal2a (Figure 9A). The remaining two galectins (PpGal3-4, JP5484429, JP549531, JP543439) share less than 39% identity to PpGal1. The novel galectins share a higher identity to related sequences from *Lu. longipalpis* (PpGal2a – 56%, PpGal3 – 87%, PpGal4 – 73%), than to mosquito galectins: *An. gambiae* (36%, 47%, 48%), *Ae. aegypti* (40%,50%, 60%) and *Cu. quinquefasciatus* (37%, 45%, 57%). The previously published galectins A-D (Dillon et al. 2006) were identified in our *Lu. longipalpis* analysis, but no additional galectins were identified (Figure 9).

Thioester-containing proteins (TEPs) are homologs of the complement system (Blandin, Levashina 2004) and have been implicated in phagocytosis of gram-negative bacteria in insects (Cirimotich et al. 2010, Tanaka et al. 2008, Blandin, Levashina 2007). Three sequences (JP540471, JP544554, JP555283) with identity to mosquito TEPs were identified in *Ph. papatasi*. One of these TEP sequences is a contig while two are singlets of short length and likely represent transcript fragments. The contig shares 98% of its amino acids with one singlet and 93% with the other, making them potentially alleles of the contig. In the *Lu. longipalpis* dataset, two sequences related to mosquito TEPs were identified, one singlet and one contig. The novel *Lu. longipalpis* TEP sequences share a 72% and 86% identity respectively to the TEP sequence identified by Dillon et al. (Dillon et al. 2006). The novel *Ph. papatasi* TEP contig has an identity of 85% and 89% to novel *Lu. longipalpis* and a 60% identity to *Ae. aegypti* TEPs. The *Ph. papatasi* TEP singlets have and identity of 89% and 86% respectively for one singlet, 78% and 87% respectively to the novel *Lu.longipalpis* TEP sequences and 69% and 53% identity to the *Ae. aegypti* TEP sequence.

Signaling pathways proteins

The Toll signaling pathway plays a key role in the establishment of the dorso-ventral axis of the *Drosophila* embryo and also is activated in response to microbial infection (Cirimotich et al. 2010, Anderson 2000). Immune responses through this pathway are activated by the recognition of a pathogen by the PGRPs that activates a serine protease cascade culminating with activation of the cytokine-like protein, Spätzle. Subsequent signaling involves Myd88, Tube and Pelle resulting in the degradation of Cactus and the release of Dorsal, a Rel1 protein, from its complex with Cactus (Anderson 2000, Michel et al. 2001). Five sequences with identity to mosquito Spätzle were identified in sand flies; three in *Ph. papatasi* and two in *Lu. longipalpis.* Two of the *Ph. papatasi* Spätzle-like sequences are non-overlapping fragments of the same transcript. The two *Lu. longipalpis* Spätzle-like have an identity of 99% to each other, making them alleles and 30% identity to the known *Lu. longipalpis* Spätzle (Dillon et al. 2006). The three novel sand fly Spätzle-like sequences have an identity of 61%, 68% and 68% to *Ae. aegypti* Spätzle proteins (Nene et al. 2007). The *Ph. papatasi* sequence identified as MyD88-like has 45% identity to *Cu. quinquefasciatus* MyD88 and is most likely a fragment, as it is short (122 AA) and a singlet. The absence of MyD88-like sequences from the *Lu. longipalpis* dataset may be explained by the lower number of sequences available for this sand fly. The four sand fly homologs to Toll-interacting protein (Tollip) (three in *Lu. longipalpis* and one in *Ph. papatasi*) have a 97% identity to each other and 68% to *Tr. castaneum* Tollip protein. Pellino, another component of the Toll pathway, is known to bind phosphorylated Pelle/IRAK and enhance innate immunity in *Drosophila* fruit flies (Haghayeghi et al. 2010). Here we identified two *Ph. papatasi* sequences with a 91% identity to the *Dr. melanogaster* Pellino sequence, however these novel sequences are from a single transcript as they are unassembled mate reads (Supplemental Table 1).

The immunodeficiency (IMD) pathway is similar to the mammalian TNF receptor signaling pathway (Valanne et al. 2007)(Lemaitre, Hoffmann 2007) and is activated by gram-negative bacteria in *Dr. melanogaster* (Tanaka et al. 2008). Several sequences with homology to components of this pathway were identified in *Ph. papatasi* including five sequences with identity to inhibitor of apoptosis protein 2 (IAP2), a protein required for antimicrobial peptide expression in fruit flies (Valanne et al. 2007). Four of the novel IAP2 sequences are most likely alleles of one protein, the fifth being rather short (96AA) representing a transcript fragment. The sand fly IAP2 sequences have an identity of 44% to *Gl. morsitans morsitans* IAP2 sequence (Attardo et al. 2006). One sequence related to IKKβ was also identified in *Ph. papatasi* (Supp Table 1).

While the first two signaling pathways are well characterized in insect species, there is less information concerning the c-Jun NH_2-terminal kinase (JNK) pathway in Diptera (Agaisse, Perrimon 2004). The JNK signaling pathway is formed by genes involved in wound repair, stress repair, and negative feedback control of antimicrobial peptides (Botella et al. 2001, Ramet et al. 2002). A component of the IMD pathway, TAK1, a MAPK, has been implicated in activation of this signaling cascade (Silverman et al. 2003). One sequence related to Hem, a component of the JNK pathway, was identified in each sand fly, but shared no significant identity to each other. Three Fos sequences were identified in the current study; 1 in *Lu. longipalpis* and two in *Ph. papatasi* with an identity of 80% between the two sand flies (the 2 *Ph. papatasi* sequences are alleles). Two sequences with identity to *Cu. quinquefasciatus* Jun sequence, another JNK pathway component, also were identified in sand flies (one each) (Supp Table 1).

Effector proteins

In insects, prophenoloxidases (PPOs) are involved in melanization, an efficient immune response activated by recognition of lipopolysacharides, peptidoglycan, and β-1,3 glucans (Soderhall, Cerenius 1998, Cerenius, Soderhall 2004). Ten sequences related to PPOs were identified in *Ph. papatasi.* Only four of these ten sequences have high identity (blastp, 10^{-50}) to related PPO sequences from other organisms. The four PPOs identified here most likely represent three unique transcripts, with PpPPO1 having two alleles (PpPPO1a (JP539467) and PpPPO1b (JP550019)). PpPPO1a,b and PpPPO2 (JP549395) have an identity of 44%, 52% and 63% respectively to *Ae. aegypti* PPO (XP_001648968.1), while PpPPO3 (JP545596) has an identity of 64% to a different *Ae. aegypti* PPO (XP_001661891.1). The reduced level of identity of the last six putative PPOs from *Ph. papatasi* could be due to their short length (the determined ORF encodes a protein less than 300 amino acids). Thirteen sequences with similarity to PPOs were identified in *Lu. longipalpis*, of these sequences, four are likely alleles of one PPO (LlPPO1) and three are alleles of a second PPO sequence (LlPPO2), with LlPPO2c allele being formed by an unassembled mate pair. The other six PPOs present in *Lu. longipalpis* share an identity of less than 64% at amino acid level to the previously mentioned *Lu. longipalpis* PPOs (LlPPO1a-d and LlPPO2a-c). The novel putative *Ph. papatasi* PPO have a varying degree of identity to their closest *Lu. longipalpis* counterpart: PpPPO1a -78%, PpPPO2 – 41% and PpPP03 – 37%.

Another type of protein implicated in immune response in mosquitoes has leucine-rich repeats (LRR) and in *An. gambiae* is involved in bacteria phagocytosis and parasite melanization (Povelones et al. 2009). Here we have identified five LRR-containing sequences in *Ph. papatasi*, two of which are likely alleles of the same gene, and two *Lu. longipalpis* sequences with an amino acid identity of 46 and 48% to *Ae. aegypti* and 68% to *Gl. morsitans morsitans* LRR- containing proteins. Lysozymes have been implicated in both digestion and immune response in mosquitoes (Ursic Bedoya et al. 2005). We identified two different lysozyme sequences from *Ph. papatasi* with identity to known insect lysozymes.

Peritrophic matrix (PM)

The PM is a non-membranous extracellular layer that surrounds the food bolus in insects. To date it has been best characterized in insects of medical or agricultural importance. In phlebotomine sand flies, there are two different types of peritrophic matrices depending on the life stage of the fly; PM type 1 (PM1) is present during the adult life stage while PM type 2 (PM2) is present during larval life stages (Marquardt et al. 2005). The synthesis and degradation of PM1 has been related to the ingestion of blood in *Lutzomyia* sand flies, with the PM being well formed 24h after a blood meal (Secundino et al. 2005). Functionally, PM1 has been implicated in both digestion (Tellam, Wijffels & Willadsen 1999, Shao, Devenport & Jacobs-Lorena 2001) and in *Leishmania*-sand fly interaction. Glutamine synthetase is involved in chitin synthesis, a major component of PM. We identified nine *Ph. papatasi* sequences related to glutamine synthetase with identity to sequences from other flies (56-96% *Lu. longipalpis;* 77-81%, *Dr. melanogaster;* 77% *Gl. morsitans morsitans;* 70-75%, *Ae. aegypti*), six of which may represent alleles of one sequence.

Three sequences with identity to *Dr. melanogaster* hemomucin, a molecule present in the fruit fly PM and involved in induction of antibacterial effector molecules (Theopold et al. 1996) were identified in *Ph. papatasi*, these sequences likely represent a single transcript with one sequence containing the signal peptide and the other two containing all the conserved glycosylation sites. Integrins are involved in cell-cell and cell-matrix interactions and have been implicated in phagocytosis of bacteria in *An. gambiae* (Moita et al. 2006). Five *Ph. papatasi* sequences were identified with high identity to β-integrins from insects with an amino acid identity between 53% for *Ae. aegypti* and 91% to *Plutella xylostella*. The length of these sequences combined with the fact they are either singlets or single mate pairs suggests that they might be fragments of β-integrin present in *Ph. papatasi.* Peritrophins are important components of insect PM (Lehane 1997), we identified one novel sequence with 71% identity to *Dr. melanogaster* peritrophin A and 25% identity to known *Ph. papatasi* peritrophins (Ramalho-Ortigao et al. 2007).

Quantitative mRNA analysis of candidate genes

Fourteen novel *Ph. papatasi* proteins were selected for an expression analysis. The sequences included the novel putative trypsin and chymotrypsin and PGRP proteins sequences were chosen for their potential role in digestion and immune response. The expression of three previously described transcripts was also analyzed (*PpTryp1*, *PpChym2* and *PpPGRP*) in order to validate our results.

Of the novel putative *Ph. papatasi* proteins, PpChym4a-12, PpTryp5a and PpPGRP-SC1 (Figure 10) are expressed at a higher level in the immature life stages, indicating that they may be involved in digestion as was shown for larval and pupal trypsin and chymotrypsin in *Ae.* aegypti (Yang, Davies 1971), where their roles were thought to be in food digestion and immune response, respectively. Previously, the expression of PpChym2 was shown to be influenced by the presence of a blood meal (Ramalho-Ortigao et al. 2003), in this study we demonstrate that while PpChym2 is expressed both in adult and immature life stages (Figure 10) it has a much higher level of expression in late larval stages than upon blood feeding (Figure 10). Unlike PpChym2, PpTryp1 is not expressed in the larval or pupal stages and, as demonstrated by Ramalho-Ortigao et al. (Ramalho-Ortigao et al. 2003) is down regulated upon blood meal (results not shown).

In blood feeding mosquitoes, there are two types of chymotrypsins that have their expression level influenced by taking a blood meal: early chymotrypsin which is highly expressed for the first hours post blood meal in *An. gambiae* (Shen, Edwards & Jacobs-Lorena 2000) and late chymotrypsin which, in *An. gambiae* has a peak expression at around

30h post blood meal (Vizioli et al. 2001). One late chymotrypsin has been previously identified in *Ph. papatasi* – PpChym2, with a peak level of expression at 30h post blood meal (Ramalho-Ortigao et al. 2003). Here we have identified two other novel putative chymotrypsins that have an expression post blood meal – PpChym13 and PpChym14a, indicating a possible implication of these proteins in blood digestion. While PpChym14a is also expressed in immature stages, though at much lower levels, PpChym13 is expressed only upon blood feeding (Figure 11 A&B).

PpPGRP and PpPGRP2 are two other *Ph. papatasi* proteins potentially involved in immune response whose expression was analyzed in this study. The previously identified PpPGRP (Ramalho-Ortigao et al. 2007, Jochim et al. 2008), is shown here to be expressed in both immature and adult life stages, with a peak of expression during larval stages, suggesting a possible involvement in larval immune response (Figure 12 A&C). PpPGRP2 has an expression profile in the larval stages similar to the PpPGRP with a peak of expression during 4th intar (L4). However, this transcript is also up regulated following a blood meal with or without (Figure 12 B&D).

Of the 71 *Ph. papatasi* sequences addressed in the study, 30 sequences share over 95% amino acid identity with another *Ph. papatasi* sequence, possibly representing allele variation. The high number of alleles present is suggestive of high genetic heterozygosity within the colony utilized for library construction in spite of the fact that this colony has been maintained under laboratory conditions since the early 1970s with substantial inbreeding. Furthermore, we developed an assembly process that favored a more compacted assembly than otherwise possible, indicating the possibility of gene duplication in *Ph. papatasi*. The existence of a high degree of polymorphism in the population is possible but further tests are necessary for confirmation.

To estimate colony genetic heterozygosity we quantified the number of SNPs present in each contig, requiring that each qualifying position was covered by at least four ESTs and that the two most common alleles were present in at least two ESTs. Using these criteria, SNPs were discovered in 1285 contigs, comprising 7.1 Mb of sequence. We identified 6312 SNPs and estimated 8.88 SNPs per 1,000 bases. This number is slightly greater than the SNP density for colonized *An. funestus* mosquitoes (Wondji, Hemingway & Ranson 2007) but lower than that found in the butterflies *Melitaea cinxia* (Vera et al. 2008) and *Papilio Zelicaon* (O'Neil et al. 2010), indicating a relatively low level of heterozygosity.

Conclusions

This work represents the first global transcriptome study of the sand fly *Ph. papatasi*, the principal vector of *Le. major* in North Africa and the Middle East, and has resulted in the identification of novel sequences involved in parasite-vector interactionthat could be targets for future vector control methods. Furthermore, this EST library will be an essential resource during annotation of the two phlebotomine sand fly genomes currently under way (McDowell et al. 2006).

Experimental procedures

Library construction

A normalized cDNA library (Soares et al. 2009) was constructed from a *Ph. papatasi* colony (Israeli strain) maintained at the Walter Reed Army Institute of Research (WRAIR). This colony was originally established in the 1970's and has been subjected to several bottlenecks, thus it is thought to display low levels of genetic polymorphism. Using a RNAeasy Mini Kit (Qiagen), total RNA was collected from the four larval instars, pupae,

and adult males and females 1, 3, and 10 days post emergence (adults fed only on sugar). Additional RNA was included from females 6, 12, 24, 36, 48, 72, 94 and 120 hours post feeding on uninfected and *Le. major* (strain Friedlin V1) infected mouse blood. A total of 29 samples were collected with RNA amounts per sample ranging from 700ng (1^{st} instar larvae or L1) to 20μg (48h post blood meal, single females). RNA samples were checked on agarose gel (1% MOPS with 5% formaldehyde). Library construction was completed with pooledRNA, using equal amounts from each of the 29 samples, by Express Genomics (Baltimore, MD) and EST sequencing was performed by the Genome Institute at Washington University (St. Louis, MO).

Sequencing, processing, and assembly

Sequences were generated from the normalized cDNA library using the Sanger-based dideoxy chain termination. Direct colony sequencing using standard high throughput sequencing methods was performed using a DNA track Robot and ABI 3730 sequencers. *Ph. papatasi* sequencing chromatograms were used to generate sequence and quality score files using Phred/Phrap program suite (Ewing et al. 1998, Ewing, Green 1998). *Lu. longipalpis* sequences and quality scores were obtained in Fasta format from Dillon et al. (Dillon et al. 2006). Cleaning and filtering of sequence files was performed in three steps. First, contaminant sequences (human, protozoa, bacteria, mouse or rat) were removed using the BLASTX algorithm (Altschul et al. 1990). A sequence was considered to be a contaminant if 12 of the first 20 best hits were any of the organisms listed above. Subsequently low quality regions were trimmed using Lucy2 (Chou, Holmes 2001) with default parameters and the pExpress-1 cloning vector. Lastly, poly(A) tails and small contaminant sequences were removed using the Seqclean algorithm available from The Institute for Genomic Research (TIGR Gene Indices Clustering Tools) (Chen et al. 2007). Only sequences longer than 100 bp were used for further analysis. As a comparison, we also analyzed a previously generated *Lu. longipalpis* EST data set (Dillon et al. 2006). To eliminate sequence cleaning and assembly biases, the *Lu. longipalpis* data (first described by Dillon et al. (Dillon et al. 2006) were processed using the same programs and parameters as the *Ph. papatasi* dataset.

Sequences were assembled into contigs using the Cap3 assembler (Huang, Madan 1999). Overlapping mate pairs were assembled and consensus obtained using relatively relaxed criteria (p 80, o 20, h 95). Consensus sequences obtained above were assembled with remaining ESTs using more stringent parameters (p 95, o 50, h 30) (Figure 1). The resulting sequences are referred to as assembled sequences.

GO annotation and similarity searches

Biological functions for the *Ph. papatasi* and *Lu. longipalpis* assembled sequences were assigned using Blast2GO (Conesa et al. 2005). InterProScan analysis also was performed as part of the GO annotation process. Annotated genes were split into the three main GO categories: biological process, molecular function and cellular component.

Similarity to known proteins

Similarity searches to known sequences were performed by using BLAST with an e-value limit of 10^{-5} against the National Center for Biotechnology Information (NCBI) non-redundant protein database (NR). Protein sequences involved in vector-parasite interactions, specifically sequences involved in blood digestion, immune response, and peritrophic matrix composition were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/protein) and ImmunoDB (http://cegg.unige.ch/Insecta/immunodb/) for available arthropod species. *Ph. papatasi* sequences with significant similarity (BLASTX, e-value 10^{-20}) to these restricted datasets were further analyzed for presence of conserved domains using ScanPROSITE (Gattiker, Gasteiger & Bairoch 2002) and manual searches. For manual domain searches,

sand fly assembled sequences were translated into amino acid sequences using prot4EST pipeline (Wasmuth, Blaxter 2004) and then aligned to known proteins using Muscle (Edgar 2004) with default parameters. Alignments were viewed using Seaview (Gouy, Guindon & Gascuel 2010) and Jalview (Waterhouse et al. 2009). Maximum likelihood trees were built for full sand fly sequences using sequences from related organisms and the MEGA5 algorithm (using the WAG model, bootstrap value: 1000) (Tamura et al. 2011).

Expression analysis of predicted *Ph. papatasi* proteins

An expression analysis was performed on 14 novel putative *Ph. papatasi* proteins involved in digestion and immune response (1 trypsin, 11 chymotrypsin, 2 PGRP). Total RNA was isolated from 5 different *Ph. papatasi* larval stages (L1-P) females and males 1-3 days old and blood fed females at 24h, using the RNeasy Mini Kit (Qiagen). For proteins with a high expression at 24h post blood meal additional samples were extracted from females at 3h, 36h and 72h post blood meal and post *Le. major* infected blood meal. The DNase (Fermentas) treated RNA was used to generate cDNA using Superscript III (Invitrogen) and oligo ($dT_{12\text{-}20}$). Quantitative PCR was performed using SYBRGreen (ABI), an ABI 7900 RT-PCR system and 20ng of cDNA per sample. Primer sets were designed for each sequence of interest such that, only one sequence was amplified (Supplemental Table 3), however, high level of identity between the potentially different alleles made it impossible to distinguish between them. The 60S Ribosomal protein L10 was used as an internal control. Reactions for each gene and for the control used were carried out in triplicate. Relative expression levels of each gene was determined by the $\Delta\Delta C_T$ method, where relative expression is expressed as a fold difference relative to sugar fed females and expressed as $2^{-\Delta\Delta CT}$. The following formula was used: $\Delta\Delta C_T = \Delta C_{T(\text{stage or condition})} - \Delta C_{T(\text{Sugar Fed Females})}$ and $\Delta C_T = C_{T\ (\text{gene of interest})} - C_{T\ (\text{60S RNA})}$. Average Ct value for all samples can be found in supplemental tables 4 and 5.

Heterozygosity Analysis—Single nucleotide polymorphisms (SNPs) were generated from the sandfly EST assemblies as described by O'Neil et al. (2010). Specifically, the ACE output of each CAP3 assembly was imported into an AMOS bank (Pop et al. 2004) for programmatic access to underlying read information. SNPs were called using the “loose” criterion, which required that the two most common alleles be found in at least two distinct ESTs (O'Neil et al. 2010). To estimate heterozygosisity, we used AMOS to count both the number of loose criterion SNPs and the number of positions covered by at least four ESTs in each contig. Average heterozygosity was then computed as the total number of SNPs divided by total number of qualifying positions. This simple method was chosen over the Beta statistic (Novaes et al. 2008) because we were not interested in population genetics underlying these two colonies and highly covered arthropod EST contigs tend to be less diverse (O'Neil et al. 2010) justifying the use of more simple SNP criteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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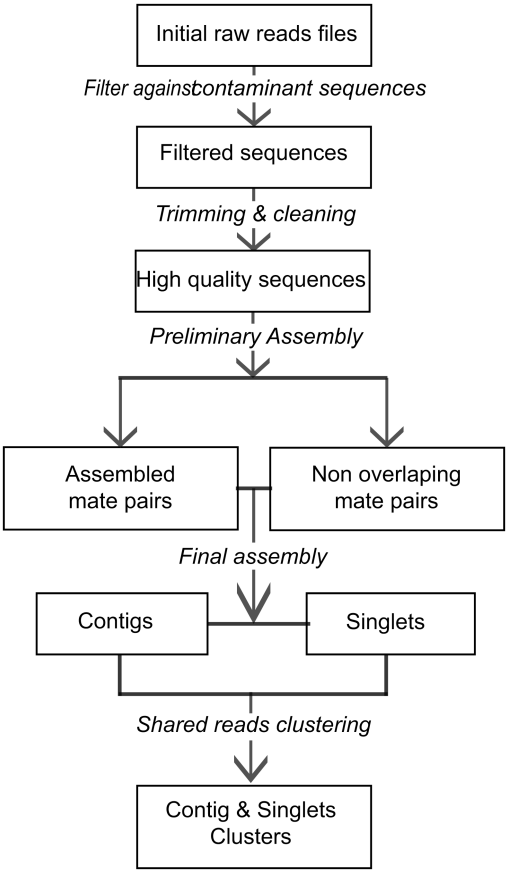


Figure 1. Assembly process flowchart

Boxes indicate a state of the sequences in the pipeline while italic lettering indicates modifications applied to the sequences and arrow indicates the sense of the sequences movement down the pipeline

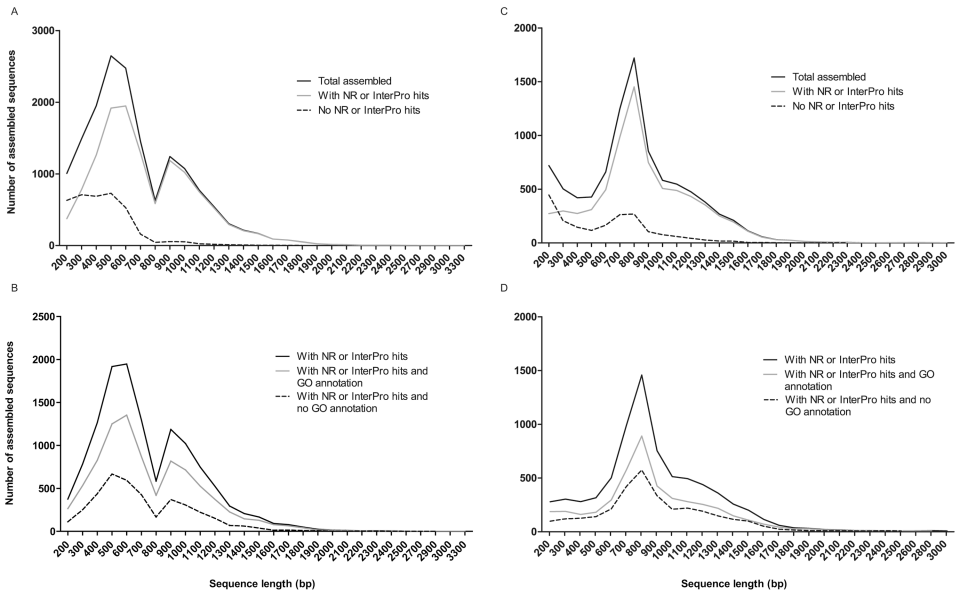
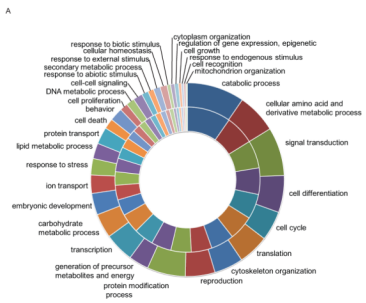
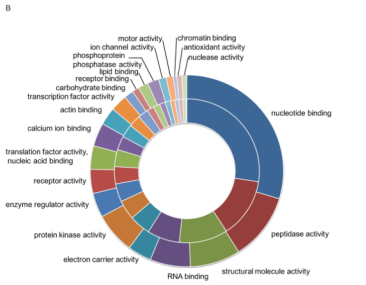


Figure 2. Assembled sequence length distribution

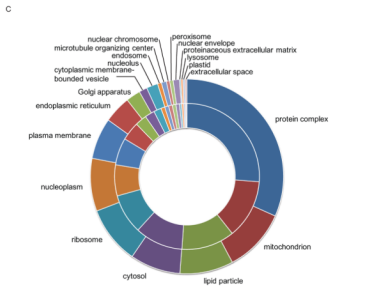
Distribution of sequence length for sequences with NR or InterPro hits and for sequences with NR or InterPro hits and GO annotation for *Ph. papatasi* (A,B) and *Lu. longipalpis* (C,D)



GO subcategory	*Phlebotomus papatasi*	*Lutzomyia longipalpis*
catabolic process	1326	562
cellular amino acid and derivative metabolic process	894	389
signal transduction	846	475
cell differentiation	813	363
cell cycle	763	290
translation	760	301
cytoskeleton organization	707	284
reproduction	629	290
protein modification process	625	376
generation of precursor metabolites and energy	610	196
transcription	598	289
carbohydrate metabolic process	594	239
embryonic development	493	212
ion transport	448	181
response to stress	417	161
lipid metabolic process	411	152
protein transport	353	166
cell death	317	101
behavior	281	130
cell proliferation	228	77
DNA metabolic process	215	82
cell-cell signaling	198	93
response to abiotic stimulus	171	65
secondary metabolic process	168	57
response to external stimulus	162	67
cellular homeostasis	122	69
response to biotic stimulus	122	35
cytoplasm organization	89	39
regulation of gene expression, epigenetic	82	39
cell growth	69	29
response to endogenous stimulus	64	21
cell recognition	57	24
mitochondrion organization	56	14
viral reproduction	12	0
symbiosis, encompassing mutualism through parasitism	6	0



GO subcategory	*Phlebotomus papatasi*	*Lutzomyia longipalpis*
nucleotide binding	1412	719
peptidase activity	689	273
structural molecule activity	558	204
RNA binding	374	163
electron carrier activity	244	97
protein kinase activity	225	167
enzyme regulator activity	209	100
receptor activity	199	98
translation factor activity, nucleic acid binding	198	96
calcium ion binding	159	95
actin binding	152	72
transcription factor activity	137	68
carbohydrate binding	120	38
receptor binding	80	28
lipid binding	76	41
phosphoprotein phosphatase activity	66	46
ion channel activity	60	31
motor activity	53	28
chromatin binding	45	15
antioxidant activity	45	21
nuclease activity	40	20
neurotransmitter transporter activity	8	0



GO subcategory	*Phlebotomus papatasi*	*Lutzomyia longipalpis*
protein complex	1898	864
mitochondrion	948	288
lipid particle	860	244
cytosol	779	231
ribosome	641	262
nucleoplasm	498	239
plasma membrane	436	187
endoplasmic reticulum	301	124
Golgi apparatus	187	66
cytoplasmic membrane-bounded vesicle	157	37
nucleolus	121	52
endosome	64	18
microtubule organizing center	64	23
nuclear chromosome	60	15
peroxisome	60	16
nuclear envelope	57	29
proteinaceous extracellular matrix	35	6
lysosome	34	10
plastid	27	9
extracellular space	16	9
external encapsulating structure	9	0

Figure 3. Gene Ontology terms distribution

Distribution of *Ph. papatasi* (inner circle) and *Lu. longipalpis* (outer circle) sequences for the three main GO categories: Biological process (A), Molecular Function(B) and Cellular Component (C).

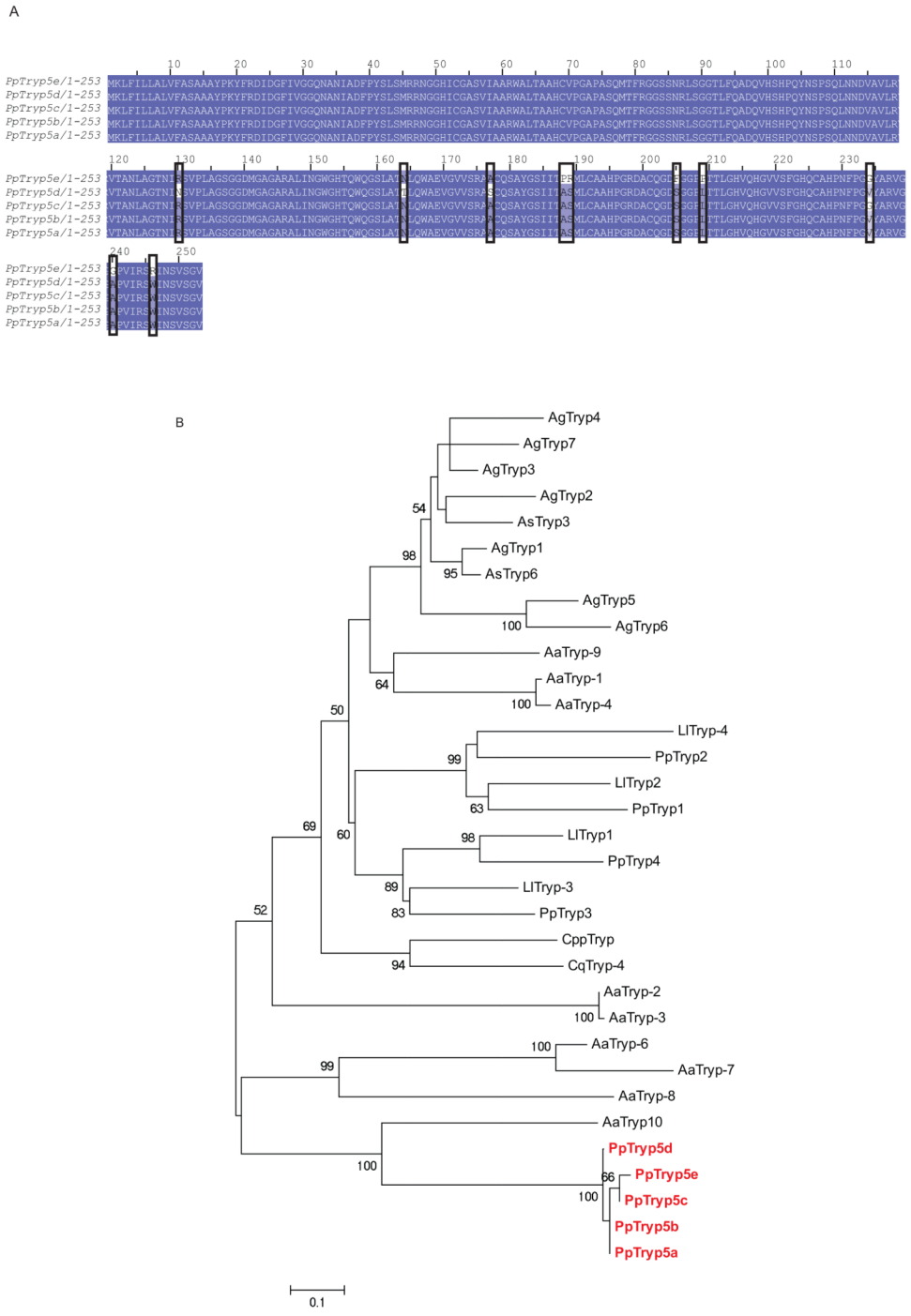


Figure 4. *Ph. papatasi* novel trypsin sequences

(A) Novel *Ph. papatasi* multiple sequence alignment; conserved residues are represented by a darker shading while mismatches between the five sequences are indicated by boxes. Full sequence alignment is available in supplemental materials (S5). (B) Phylogenetic analysis of trypsin amino acid sequences from *Ph. papatasi* (Pp: PpTry5a-e (JP544502, JP542407, JP540627, JP554453, JP544448), AAM96940.1, AAM96941.1, AAM96942.1, AAM96943.1), *Lu. longipalpis* (Ll: ABM26904.1, ABM26905.1, ABV60308.1, ABV60300.1), *An. gambiae* (Ag: CAA80512.1, CAA79328.1, CAA80517.1, CAA80515.1,

CAA80514.1, CAA80513.1, CAA80516.1), *An. stephensi* (As: AAB66878.1, AAA97479.1), *Ae. aegypti* (Aa: EAT40684.1, EAT42808.1, EAT36350.1, EAT34033.1, EAT42007.1, EAT42008.1, EAT42004.1, EAT37859.1), *Cu. quinquefasciatus* (Cq: EDS34988.1) and *Cu. pipiens palens* (Cpp: AAK67462.1). The WAG substitution model was used with variable positions and a bootstrap value of 1000 (only those above 50 are represented on the trees). The scale represents the rate of amino acid substitution per site. The novel *Ph. papatasi* trypsin sequences (PpTryp5a-e) are indicated in bold.

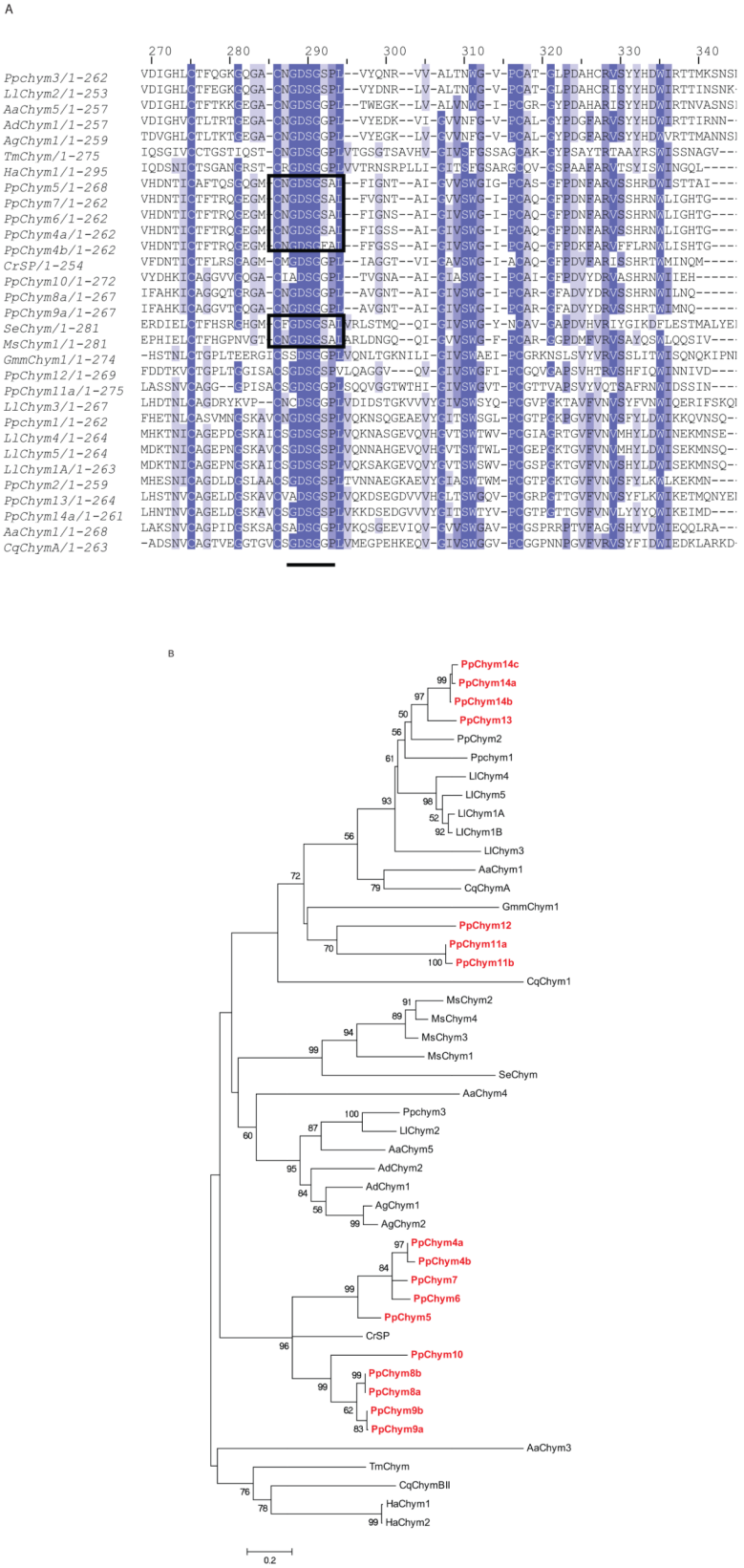


Figure 5. *Ph. papatasi* novel chymotrypsin sequences

(A) Multiple sequence alignment fragment depicting the chymotrypsin binding pocket, where the pockets with the P-to-A substitution are boxed; the residue concordance at each position in indicated by the different degree of shading at that position. Full sequence alignment is available in supplemental materials (S6). (B) Phylogenetic analysis of known and novel chymotrypsins from *Ph. papatasi* (Pp: PpChym4-14 (JP546634, JP554565, JP551370, JP547341, JP554731, JP549601, JP540150, JP554746, JP554644, JP540516, JP543908, JP549141, JP547864, JP543702, JP542007, JP548909, JP549999), AAM96938.1, AAM96939.1, ABV44728.1), *Lu. longipalpis* (Ll: ABV60294.1, ABV60293.1, ABV60309.1, ABV60291.1, ABV60292.1, ABV60301.1), *An. gambiae* (Ag: CAA83568, CAA83567), An. darlingi (Ad: ADD17493.1, ADD17494.1), *Ae. aegypti* (Aa: XP_001663061.1, EAT32679.1, EAT38422.1, AAL93243.1), *Cu. quinquefasciatus* (Cq: XP_001846630.1, XP_001865429.1, XP_0011863473.1), *Ch. riparius* (Cr: ACF19792.1), *Gl. morisitans morisitans* (Gmm:ADD18377.1), *He. armigera* (Ha: ADI32883.1, ADI32881.1), *Ma. sexta* (Ms: CAL92020.1, CAM84317.1, CAM84318.1, CAM84319.1), *Sp. exigua* (Se: AAX35812.1) and *Te. molitor* (Tm: ABC88746.1). The WAG substitution model was used with variable and invariable positions and a bootstrap value of 1000 (only those above 50 are represented on the trees). The scale represents the rate of amino acid substitution per site. The novel *Ph. papatasi* chymotrypsin sequences are in bold.

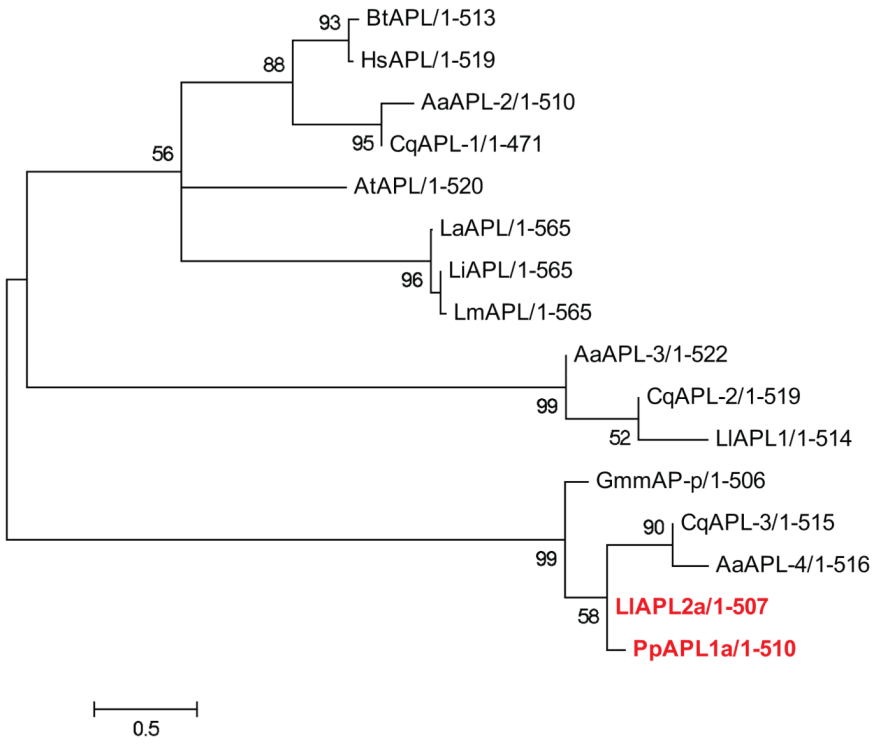


Figure 6. Phylogenetic analysis of *Ph. papatasi* and *Lu. longipalpis* aminopeptidase L

Philogenetic tree constructed using aminopeptidase L sequences from *Ph. papatasi* (Pp: PpAPL1a (JP541859)), *Lu. longipalpis* (Ll: LlAPL1, LlAPL2a), *Ar. thaliana* (At: CAA45040.1), *Bo. taurus* (Bt: AAB28170.1), *Gl. morisitans morisitans* (Gmm: ADD18517.1), *Ae. aeygpti* (Aa: EAT48532.1, EAT47208.1, EAT45789.1), *Cu. quinquefasciatus* (Cq: XP_001866727.1, XP_001844372.1, XP_001851548.1), *Le. amazonensis* (La: AAL16095.1), *Le. infantum* (Li: CAM68214.1), *Le. major* (Lm: XP_001683430.1), *Homo sapiens* (Hs: AAD17527.1) and *Bo. mori* (Bm: NP_001108470.1). The WAG substitution model was used with variable and invariable positions and a bootstrap value of 1000 (only those above 50 are represented on the trees). The scale represents the rate of amino acid substitution per site. Novel sand fly aminopeptidase L sequences are in bold.

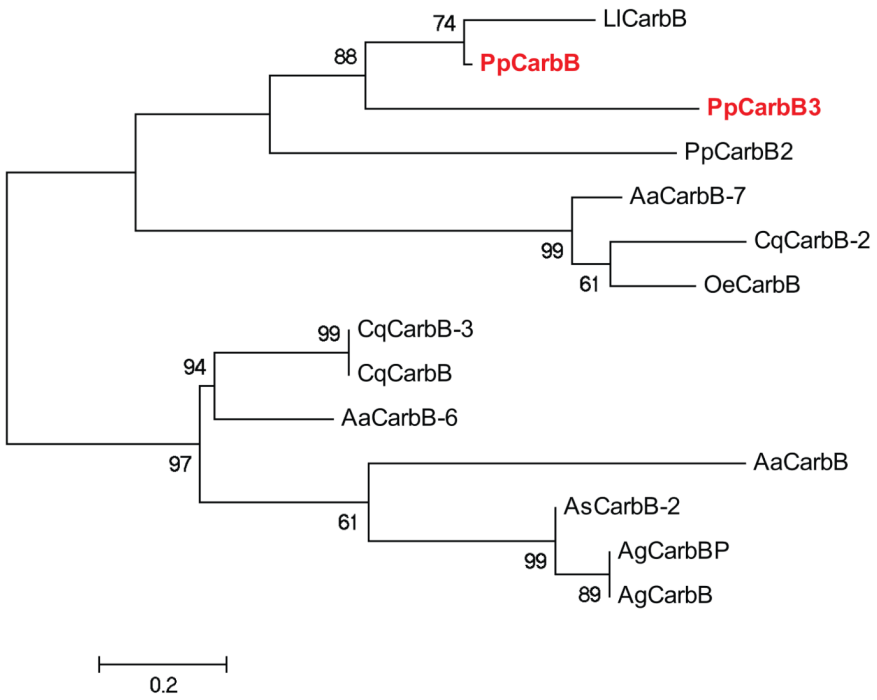


Figure 7. Phylogenetic analysis of *Ph. papatasi* and *Lu. longipalpis* carboxypeptidase B

Phylogenetic tree constructed using carboxypeptidase B sequences from *Ph. papatasi* (Pp: PpCarbB2 (JP542144), PpCarbB3 (JP546271), ABV44754.1), *Lu. longipalpis* (Ll: LlCarbB), *An. gambiae* (Ag: AAS99341.1, CAF28572.1), *An. stephensi* (As: ADD31639.1), *Ae. aeygpti* (Aa: AAT36733.1, AAT36732.1, ABO21077.1), *Cu. quinquefasciatus* (Cq: XP_001856154.1, EDS34658.1, XP_001856164.1), *Oc. epactius* (Oe: AAT36738.1). The WAG substitution model was used with variable and invariable positions and a bootstrap value of 1000 (only those above 50 are represented on the trees). The scale represents the rate of amino acid substitution per site. Novel *Ph. papatasi* carboxypeptidase B sequences are in bold.

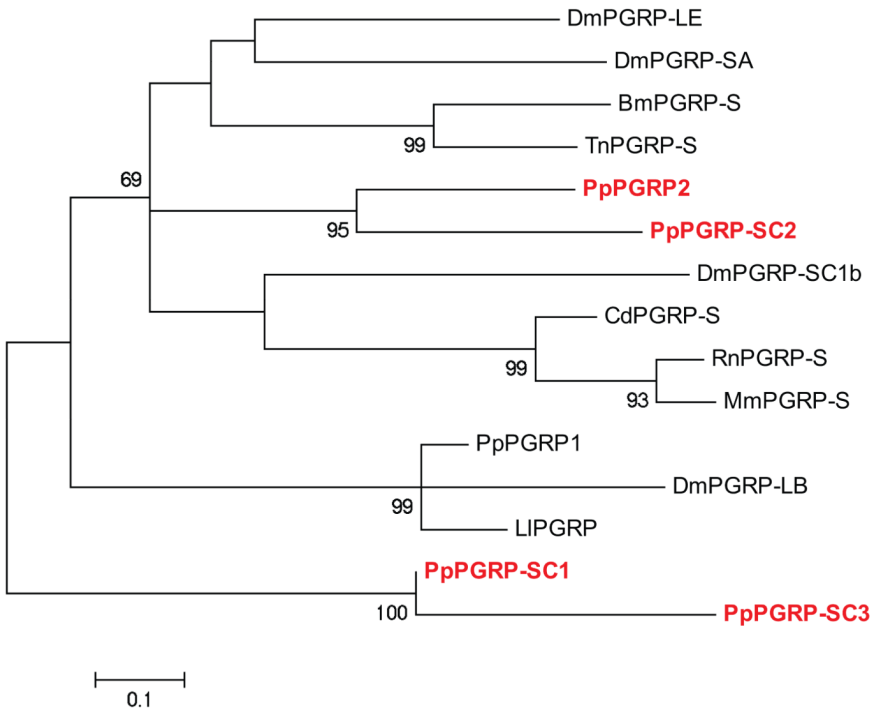


Figure 8. Phylogenetic analysis of *Ph. papatasi* PGRP sequences

Phylogenetic analysis of known and novel *Ph. papatasi* PGRP sequences from *Ph. papatasi* (Pp: ABV60369.1, PpPGRP2 (JP540873), PpPGRP-SC1-3 (JP551327, JP547206, JP546057), *Lu. longipalpis* (Ll: ABV60332.1), *Bo. mori* (Bm: BAA77209.1), *Ca. dromedaries* (Cd: CAC19553.1), *Dr. melanogaster* (Dm: AAF54643.1, AAG32064.1, AAG23735.1, AAG23736.1), *Mu. musculus* (Mm: AAC31821.1), *Ra. norvegicus* (Rn: AAF73252.1) and *Tr. ni* (Tn: AAC31820.1). The WAG substitution model was used with variable positions and a bootstrap value of 1000 (only those above 50 are represented on the trees). The scale represents the rate of amino acid substitution per site. Novel *Ph. papatasi* PGRP sequences are in bold.

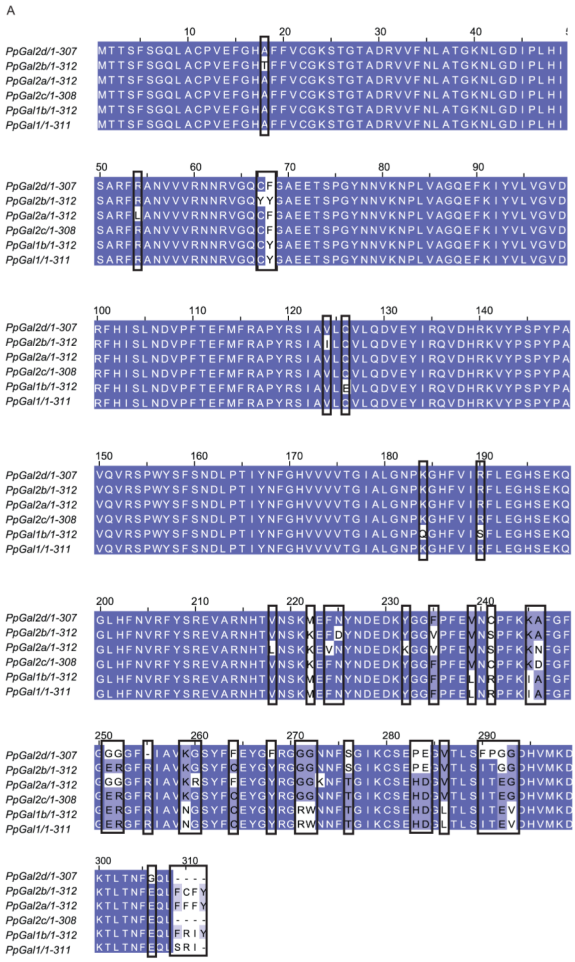


Figure 9. *Ph. papatasi* novel galectin sequences

Multiple sequence alignment of five novel *Ph. papatasi* galectins (PpGal1b (JP539352), PpGal2a-d (JP540648, JP546602, JP550066, JP540193) and the known galectin sequence (AAT11557.1) from the same organism. Darker shading indicates conservation at each position.

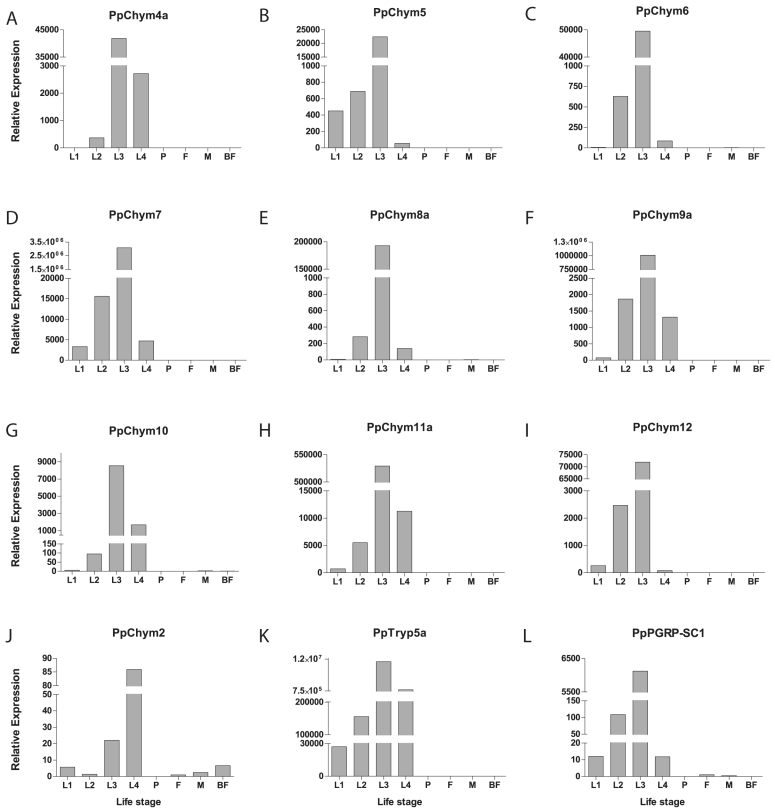


Figure 10. *Ph. papatasi* novel proteins transcript levels

qPCR analysis of *Ph. papatasi* novel putative genes at different life stages, including larvae (L1-L4), pupa (P), 1-3 days past emergence adult females (F) and males (M) and adult females at 24h post blood meal (BF) for (A) PpChym4a (JP546634), (B) PpChym5 (JP551370), (C) PpChym6 (JP547341), (D) PpChym7 (JP554731), (E) PpChym8a (JP549601), (F) PpChym9a (JP554746), (G) PpChym10 (JP540516), (H) PpChym11a (JP543908), (I) PpChym12 (JP547864), (J) PpChym2 (AY128107), (K) PpTryp5a (JP544502) and (L) PpPGRP-SC1 (JP551327).

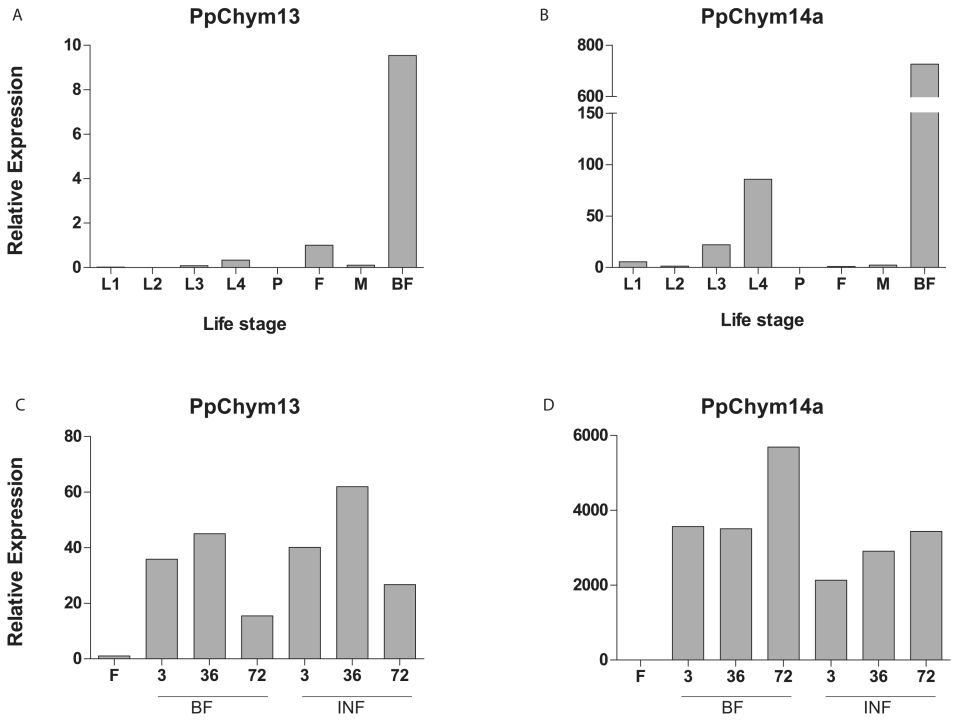


Figure 11. PpChym13 and PpChym14 transcript levels

qPCR analysis of 2 *Ph. papatasi* novel putative chymotrypsins at different life stages, including larvae (L1-L4), pupa (P), 1-3 days past emergence adult females (F) and males (M) and adult females at 24h post blood meal (BF) for (A) PpChym13 (JP543702) and (B) PpChym14a (JP542007). Additional blood feeding conditions for adult female sand flies at 3h, 36h and 72h post blood meal (BF) and post *Le. major* infected blood meal (INF) for (C) PpChym13 and (D) PpChym14a.

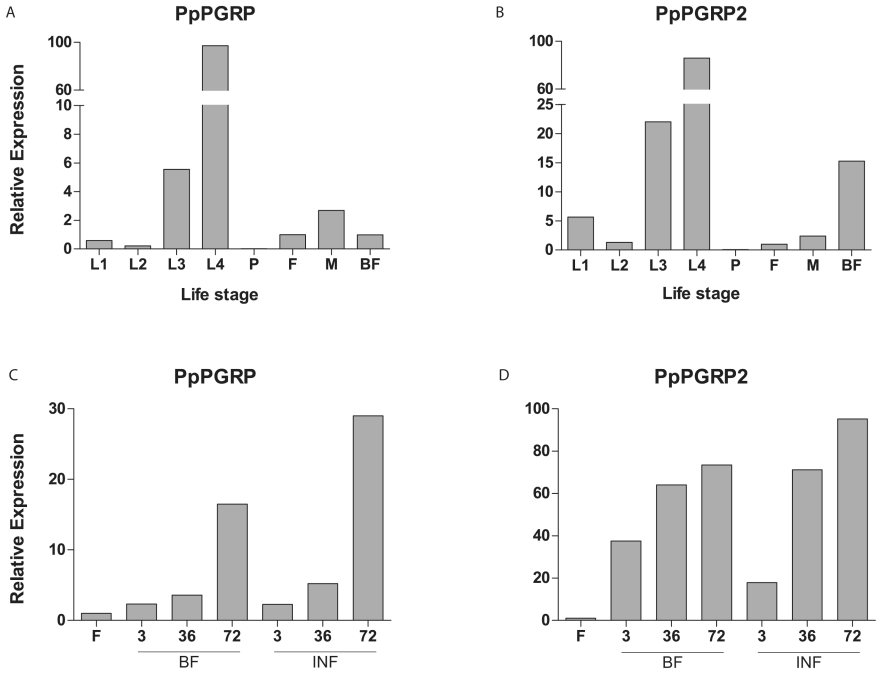


Figure 12. PpPGRP and PpPGRP2 transcript levels

qPCR analysis of 2 *Ph. papatasi* novel putative PGRPs at different life stages, including larvae (L1-L4), pupa (P), 1-3 days past emergence adult females (F) and males (M) and adult females at 24h post blood meal (BF) for (A) PpPGRP (EU130784) and (B) PpPGRP2 (JP540873). Additional blood feeding conditions for adult female sand flies at 3h, 36h and 72h post blood meal (BF) and post *Le. major* infected blood meal (INF) for (C) PpPGRP and (D) PpPGRP2.

Table 1

Assembly results for *Phlebotomus papatasi* and *Lutzomyia longipalpis* at all steps of the assembly process presented in Fig. 1.

Assembly steps		*Phlebotomus papatasi*	*Lutzomyia longipalpis*
Initial sequences	Total	47,615	27,928
	Normalized	47,615	26,495
	Non-normalized	N/A	1,433
Filtering	Passed	47,227	27,863
	Failed	388	65
Cleaning and trimming	Lucy	37,708	24,102
	SeqClean	37,487	24,019
First tier assembly	Contigs	7,683	6,049
	Singlets	22,121	11,101
Second tier assembly	Contigs	6,187	5,063
	Singlets	10,933	4,963
Total		17,120	10,026
Sequences longer than 200 nucleotides		16,265	N/A